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USE OF A SENSITIVE MICROPLATE ENZYME-LINKED IMMUNOSORBENT ASSAY IN A RETROSPECTIVE SEROLOGICAL ANALYSIS OF A LABORATORY POPULATION AT RISK TO INFECTION WITH TYPHUS GROUP RICKETTSIAE.

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Use of a Sensitive Microplate Enzyme-Linked Immunosorbent Assay in a Retrospective Serological Analysis of a Laboratory Population at Risk to Infection with Typhus Group Rickettsiae†

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A microplate enzyme-linked immunosorbent assay (ELISA), developed for the detection of antibodies to typhus group rickettsiae, was used to analyze human sera from individuals engaged directly or indirectly in rickettsial research. The earliest serum available from each of 112 individuals was tested for immunoglobulin M (IgM) and IgG antibodies against *Rickettsia typhi* and *Rickettsia prowazekii* by ELISA at a 1:500 dilution. In at least one assay, nine sera had ELISA optical densities of >0.2, which were above the mean optical densities plus three standard deviations of the other 103 sera. Three of the positive sera were from individuals with known clinical cases of typhus infection. The other sera with predominantly IgG titers were from individuals with extended laboratory exposure to rickettsiae or histories of typhus vaccination, or both. During continued serological surveillance, eight additional people with repeated occupational exposure to typhus rickettsiae had seroconversions in the ELISA to optical densities of >0.2. No apparent clinical illness occurred in two individuals, whereas six clinical cases of infection occurred in others subsequent to accidental laboratory autoinoculation (one) or aerosol exposures (five). In the clinical infections, antibodies were first detected at 7 days, but in subsequent sera, rises and declines in titers were quite variable and were influenced by vaccination, relapse, and time and extent of antibiotic therapy. In primary infections the sera of several individuals who received immediate antibiotic therapy had brief strong IgM responses without pronounced increases in IgG. In contrast, much higher IgG levels were attained in three cases in which relapse occurred, the individual had previously been immunized, or treatment had been delayed. The microplate ELISA proved to be a highly sensitive and reliable test for detection of the human serological response to typhus antigens.

Although endemic rickettsial diseases remain a major health problem in much of the world, in the United States and Europe laboratory personnel presently constitute a major portion of the population at risk to infection with Q fever, typhus, and scrub typhus rickettsiae. In a summary and analysis of nearly 4,000 laboratory-associated infections which have come to his attention through 1974, Pike (22) lists rickettsiae as contributing approximately 15% of the total number of cases. *Coxiella burnetii* accounted for about 48% of the rickettsial infections; typhus rickettsiae (*Rickettsia prowazekii* and *Rickettsia typhi*), 32%; *R. rickettsii*, 11%; and the remaining rickettsiae, 9%. Although some cases were due to recognized accidents, such as autoinoculation or ectoparasite bite, the majority were attributed to aerosol exposure. Some of the laboratory infections which occurred before or after 1974 were well documented, as, for example, Q fever (4, 11, 13, 25), typhus (1, 36, 38), Rocky Mountain spotted fever (3, 12, 20, 30), and scrub typhus (38). Many, however, were reported to Pike (22) but were not published, and many more, undoubtedly, were not reported. Oster et al. (20) obtained immunological evidence of exposure in the absence of clinical disease, but in general, this type of information is fragmentary.

Accurate and efficient serological procedures are essential for the correct estimation of the prevalence of infectious disease in a population. Although the full spectrum of serological methods has been used in disease surveillance, the enzyme-linked immunosorbent assay (ELISA) is achieving a prominent place in these studies

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because it is highly specific, sensitive, and very flexible (28, 34). The ELISA can be used as a manual semiquantitative procedure suitable for field conditions (32) or as a fully automated quantitative method in a demanding high-volume central processing laboratory (26, 27). To survey large numbers of sera obtained from populations where rickettsial disease is endemic (15, 31), we have described the purification of stable rickettsial antigens which were suitable for use in sensitive ELISAs for antibodies against typhus and scrub typhus rickettsiae (5, 10). We describe here the application of a microplate ELISA for antibodies against typhus rickettsiae to a retrospective serological analysis of laboratory workers exposed to murine and epidemic typhus rickettsiae, using more satisfactory antigen preparations. A large number of laboratory personnel working with a variety of aerobic and anaerobic gram-negative pathogenic bacteria and various administrative personnel working adjacent to the microbiology laboratories served as the control group. The ELISA proved to be invaluable for the recognition of subclinical infections and for the evaluation of the effects of prior immunization and variations in antibiotic therapy on the typhus antibody response of those persons who had experienced clinical typhus illness.

MATERIALS AND METHODS

Microplate ELISA for antibodies against typhus rickettsiae. *R. typhi* (Wilmington strain) and *R. prowazekii* (Breinl strain) were purified from frozen pools of infected yolk sacs of embryonated eggs by differential centrifugation, isopycnic banding in two cycles of Renografin density gradients, and filtration through a glass depth filter (AP-20, Millipore Corp., Bedford, Mass.) as described previously (6, 33). The final rickettsial suspension was washed twice in K36 buffer (33), and individual preparations were suspended in 0.01 M $NaPO_4$ buffer, pH 7.0, or saline at 0.7 to 3.0 mg of protein per ml. In initial experiments, we used the classical ether-extracted saline-soluble typhus antigen that we described previously for the tube method ELISA (10). Because of the low yield of antigen obtained by this technique and the possibility that the ether extraction might have degraded or might have been selective for certain rickettsial antigens, we tested two other antigen preparations. The rickettsiae were (i) passed through an Aminco French pressure cell twice at 20,000 lb/in^2 or (ii) sonically treated at 100-W output five times in an ice bath for 30 s, followed each time by a cooling interval of 60 s, using a model W185 Sonifier Cell Disruptor (Branson Sonic Power Co., Danbury, Conn.). Each fraction was then centrifuged at 12,000 rpm for 15 min in a Sorvall SS-34 rotor (17,400 × g) to pellet intact cells and crude debris. Formalin was added to a final concentration of 0.1% to each fraction, and the antigens were stored at 4°C. The immune and hyperimmune rabbit antisera used previously in the typhus tube method ELISA (10) were again used here to standardize the typhus antigens in the microplate ELISA procedure, which was identical to that described previously for *Rickettsia tsutsugamushi* antigens (5).

The optimal coating dilution of each antigen was defined as that dilution giving a maximum optical density (OD) with a background control (no serum) of <0.15 OD. Although each of the antigens prepared from purified rickettsiae could be used in the microplate ELISA, the optimal coating concentrations did not differ greatly (1.33 to 6.27 μg/ml; Table 1). Although the recovery of rickettsial protein after ether extraction or sonication was greater in 0.01 M $NaPO_4$ than in saline (not shown), yields were uniformly reproducible and greatest after pressure cell disruption of the rickettsiae in either diluent (Table 1). The higher ODs attained at the optimal coating dilution with the pressure cell-extracted antigen also permitted the most sensitivity in determining antibody titers (Table 1). The 0.01 M $NaPO_4$ pressure cell extracts were very stable since no changes were observed in their ELISA properties after storage at 4°C for 2 years. For these reasons, the latter pressure cell extract antigens were selected for the serological survey of human sera.

Micro-IF test. Bovine plasma albumin-purified rickettsiae (step 2, reference 33) were used, and the microimmunofluorescent antibody test (micro-IF) was performed as described previously for *R. tsutsugamushi* (5).

RESULTS

Comparison of serum titers by micro-IF and ELISA. Endpoint titers were determined by both micro-IF and ELISA for a set of 12 sequential sera from two clinical cases of *R. typhi* infection. The correlation was excellent (r = 0.93 for immunoglobulin M [IgM]; r = 0.84 for IgG), confirming the comparability of the methods noted previously with scrub typhus antigens (5). The micro-ELISA was about 0.5 log more sensitive than the micro-IF ($P < 0.01$) with each immunoglobulin class, and although neither assay was highly specific for *R. typhi* or *R. prowazekii*, titers were significantly greater ($P <$ 0.05) against *R. typhi* by 0.15 to 0.3 log dilution (not shown; cf. Table 1).

Identification of laboratory personnel with antibodies against typhus rickettsiae by microplate ELISA. An initial retrospective survey by ELISA for IgM and IgG antibodies against *R. typhi* and *R. prowazekii* was made on the earliest serum available from each of 112 individuals who had been associated with a microbiology department during the past several years. Thirty-five of these individuals had been engaged in rickettsial research or had had significant contact with these rickettsial agents. A preliminary survey of a subset of the sera at a 1:100 dilution often gave relatively high OD values

TABLE 1. *Characterization of different typhus group antigen preparations by microplate ELISA*

Antigen prepn	Protein recovery[a] (%)	ELISA antigen dilution		ELISA rabbit antiserum titer[b]			
				R. typhi		*R. prowazekii*	
		Protein[c] (μg/ml)	OD[d]	No. 7	No. 8	No. 10	No. 12
R. prowazekii							
Ether	13	1.33	0.69	8,500	7,700	21,000	20,000
Pressure cell	76	2.42	0.82	26,500	22,000	46,000	34,000
Sonic extract	62	6.27	0.69	10,800	19,500	23,500	29,500
R. typhi							
Ether	28	2.37	0.81	16,200	24,000	12,700	11,000
Pressure cell	70	1.88	0.96	27,000	24,000	19,000	9,600
Sonic extract	57	1.54	0.83	21,000	27,000	19,500	14,000

[a] Initial whole cells = 100.

[b] Endpoint titer is the reciprocal of the greatest serum dilution with an OD 0.25 above controls without serum. Goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate was used.

[c] Antigen concentration in microplate ELISA with greatest OD with *R. typhi* rabbit no. 7 at a 1:4,000 dilution, and background control without serum no greater than 0.15 OD.

[d] OD (400 nm) at optimum antigen dilution.

which did not appear to be specific. Therefore, a 1:500 serum dilution was used for the initial screening. To exclude borderline positives that may be due to occasional aberrant nonspecific reactions, we have defined seroconversion for typhus antibodies as those cases in which ODs of >0.2 were observed in one of the four ELISA parameters, i.e., IgM and IgG against both *R. typhi* and *R. prowazekii*, in at least two successive sera when tested at a 1:500 dilution.

The ELISA OD values (minus control without serum) for 103 sera were normally distributed for each of four parameters examined (Fig. 1a to d). From the combined data of the normally distributed sera, a mean of 0.05 OD and standard deviation of 0.04 OD was calculated with relatively small variation among the four panels. In Fig. 1a, seven sera had ELISA OD values of >0.2, which is more than three standard deviations above the mean. Sera designated 1 and 2 were late acute sera from two unvaccinated but occupationally exposed individuals with previously diagnosed clinical laboratory infections. Sera 3 and 4 were obtained from two individuals who also had occupational exposure to typhus rickettsiae. Individual 3 had spent considerable time in areas endemic for both murine and epidemic typhus and had received six doses of Cox-type epidemic typhus vaccine between 1961 and 1965. Individual 4 had received a single vaccine dose in 1945. All four sera had elevated OD readings for both IgG and IgM antibodies with both antigen preparations. Since it seemed unlikely that the high IgM responses of sera 3 and 4 were due entirely to old vaccinations, these individuals probably had unrecognized (subclinical?) infections at some more recent time. Sera 5 and 6 were obtained from individuals with histories of Cox-type vaccine immunizations and 25 to 30 years of laboratory exposure to rickettsiae. Both individuals had significant IgG antibodies to both antigens but no IgM antibodies. Neither had experienced a clinical case of typhus infection. Serum 7 was also derived from an individual with extensive laboratory experience with rickettsiae, who, in addition, had a clinically confirmed case of infection with the Wilmington strain of *R. typhi* 18 years previously. This serum, as well as all other sera obtained from this person, was unusual in its very high degree of species specificity in the ELISA (cf. Fig. 1a and b). Serum 7 did not have measurable IgM antibodies against either antigen. Two additional sera had IgG ODs against *R. prowazekii* of slightly greater than 0.2 (Fig. 1b). One individual, a military dependent, had resided in the Middle East and probably had received typhus vaccine. The other individual was occupationally exposed to rickettsiae but had no history of infection or vaccination. Among the initial group, by criteria described above, individuals 1 to 7 exhibited seroconversion, whereas the last two cases could not be confirmed because no other sera were available for those individuals.

Tests of subsequent serum samples from this group and from 13 additional staff members who were not working with rickettsiae but who were still in the department 1 year after the initial survey confirmed the above results. Only one serum exceeded the 0.2 OD cutoff at a 1:500 serum dilution with an *R. typhi* IgM OD value of 0.21 (not shown). In all, without counting confirmatory tests, a total of 356 assays were performed on sera from 90 individuals with no known rickettsial exposure. Only one assay yielded a value above the positive typhus OD

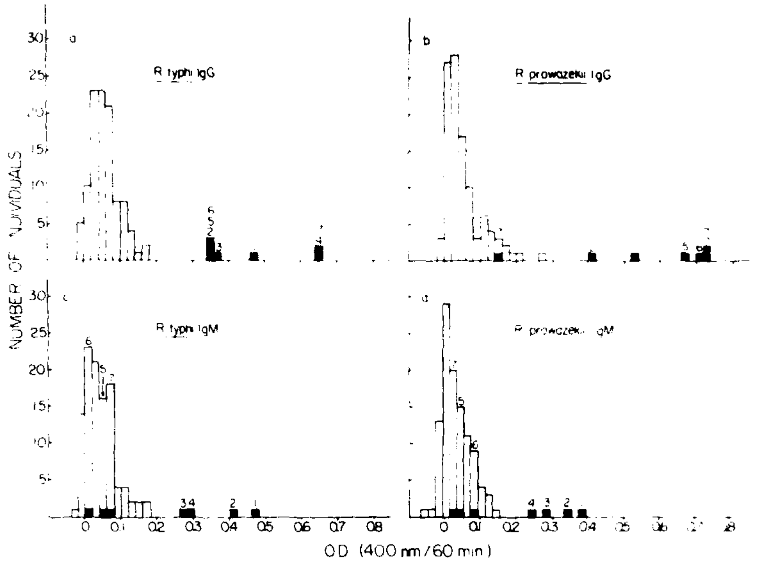

FIG. 1. *Distribution of ELISA OD values for typhus group antibodies in an initial survey of 112 laboratory personnel. All sera were diluted 1:500. The source of the sera numbered 1 to 7 is described in the text. Goat anti-human IgM and IgG immunoglobulin-alkaline phosphatase conjugate was used.*

cutoff.

Among the 35 initial staff members involved with rickettsiae, additional sera were obtained from 19 individuals with first sera negative for typhus antibodies. Over a period of 4 years, eight individuals showed seroconversion, as defined above. Six had clinical cases of infection with *R. typhi*, one by accidental autoinoculation and five presumably by aerosol exposure, whereas the other two individuals had no known histories of clinical illness. Among the 22 sera from the remaining 11 individuals, all but 1 serum had the same OD distribution as obtained with the negative control sera from nonexposed individuals (Fig. 1) for all four tests. The positive serum had IgG ODs of 0.21 and 0.26 with *R. typhi* and *R. prowazekii*, respectively, but none of three other sera from this individual was positive. Interestingly, this person had a positive lymphocyte transformation response against rickettsial antigens (2).

Antibody response to typhus rickettsiae as measured by the microplate ELISA. ELISA results were obtained on 76 sera from 15 individuals whose sera had shown seroconversion in the ELISA for typhus antibodies. In spite of the heterogeneity of the histories of these sera, limited analyses of the responses can be made (Table 2). First, when the ELISA ODs at a 1:500 serum dilution were compared with the endpint titers (greatest serum dilution with an OD 0.25 above background), excellent correlations (r = 0.76 to 0.98) were obtained. Second, it was apparent that a large number of acute and convalescent sera did not have positive ELISA ODs, particularly when the IgM conjugate was used. All of these negative IgM results, except one, were from sera late in convalescence when IgG was still present. The exception was a delayed heterologous response with *R. prowazekii* antigen. Negative IgG ODs were evenly distributed between early acute sera with IgM titers and late convalescent sera from *R. typhi* infections, with heterologous serum titers against *R. prowazekii* falling more rapidly than IgG *R. typhi* titers. In only one *R. typhi* infection (case 1, Fig. 1) did all four ELISA parameters fall below significance at 20 months postinfection, although several other unvaccinated infected individuals, treated promptly and vigorously with tetracycline, had very low IgG levels by 3 months postinfection with *R. typhi* (Fig. 2). In contrast, the vaccinated individuals (cases 3, 4, 5, and 6, Fig. 1), who possibly also had unrecognized rickettsial infections, generally retained significant persisting IgG levels against both antigens, but particularly against *R. prowazekii*. The three negative *R. prowazekii* IgG titers in the old exposure group (Table 2) were from the previously discussed specific *R. typhi*-infected individual (Fig. 1, case 7). In four infections sera were drawn at sufficiently close intervals to iden-

TABLE 2. *Analysis by ELISA of antibody response of 15 individuals to rickettsial antigens*

Antigen	Antibody class	Total no. of sera	Distribution of sera: Negative: Early sera[c]	Distribution of sera: Negative: Late sera[d]	Distribution of sera: Negative: Old exposure[e]	Distribution of sera: Positive (%)	Day of initial response[a]	OD value vs end-point titer[b] (correlation coefficient)
R. typhi	IgG	76	1	1	0	74 (97)	7–10	0.94
	IgM	67	0	13	15	39 (58)	7–10	0.82
R. prowazekii	IgG	76	6	7	3	60 (79)	7–23	0.98
	IgM	67	1	16	16	34 (51)	7–10	0.76

[a] Based on four infections during which sequential sera were drawn at sufficiently close intervals.
[b] Based on sequential sera from four infections (see Fig. 2).
[c] <15 days postonset.
[d] >3 months postinfection and treatment.
[e] Prior to initiation of this study.

tify day 7 postonset as the earliest day of significant antibody response detectable by the described assay method (Table 2).

Effects of antibiotics and prior immunization on the antibody response to infection with typhus rickettsiae. The sera of the individuals who experienced an accidental infection were further characterized by ELISA end-point titrations. Representative patterns of antibody response are shown in Fig. 2. Figure 2a illustrates a case typical of four clinical infections with *R. typhi* which were recognized early and treated promptly and vigorously with tetracycline. An early rise (within 7 days) in IgM titer against the homologous strain was followed almost immediately by a more limited rise in IgG antibodies. There was a slight delay in the more moderate response of both IgM and IgG antibodies against *R. prowazekii*. Whereas IgG titers were remarkably transient and never exceeded the IgM titers, both titers had markedly declined by 3 months after onset. Figure 2b illustrates the *R. typhi* infection of an individual on whom a short course of doxycycline treatment was used and who experienced a relapse 12 to 13 days postonset of illness. In contrast to the case in Fig. 2a, relatively high IgG titers were attained which persisted for at least 38 months. Figure 2c illustrates the antibody response to an *R. typhi* infection in an antibiotic-treated individual who had been previously vaccinated with the commercial epidemic typhus vaccine. An immediate IgG response with sustained high titers against both antigens was observed, but in contrast to the primary cases in Fig. 2a and b, the IgM response was delayed and quite low. Figure 2d illustrates the response of an individual in whom the diagnosis of rickettsial infection and consequent antibiotic treatment was delayed to 6 days postonset of symptoms. Both IgM and IgG antibodies to both antigens rose rapidly to high levels, and the IgG titers have remained high for at least 16 months after infection.

DISCUSSION

The typhus group of rickettsiae clearly presents a risk of infection to laboratory workers. Of 35 workers involved with rickettsiae, 2 had suspect low positive ODs in only one assay of a single serum sample (Fig. 1b), 15 exhibited antibody seroconversion, and another antibody suspect individual was confirmed positive by lymphocyte transformation (2). Nine workers had clear episodes of clinical typhus illness, seven apparently with *R. typhi* and two in which the causative species, by both serological and epidemiological data, was ambiguous. One of those infected with *R. typhi* had been immunized with Cox-type *R. prowazekii* vaccine. Five of the remaining seven antibody-positive individuals had been vaccinated with epidemic typhus vaccine, whereas the other two had worked extensively with the live vaccine E strain of *R. prowazekii*; all seven had higher titers to *R. prowazekii* than to *R. typhi*. The latter two individuals, as well as two of the vaccinees, had no episodes of febrile typhus-like illness or antibiotic therapy and possibly had completely inapparent infections. The other three had ill-defined histories of febrile illness which had not been treated as rickettsial infections to our knowledge. Similar suggestions of inapparent infections have been reported in laboratory workers exposed to Rocky Mountain spotted fever (20). However, the term "inapparent infection" is always subjective and hard to evaluate because of possible undocumented illness, immunization, antibiotic therapy, or exposure to related agents.

The tube and microplate ELISAs for detecting antibodies against typhus and scrub typhus rick-

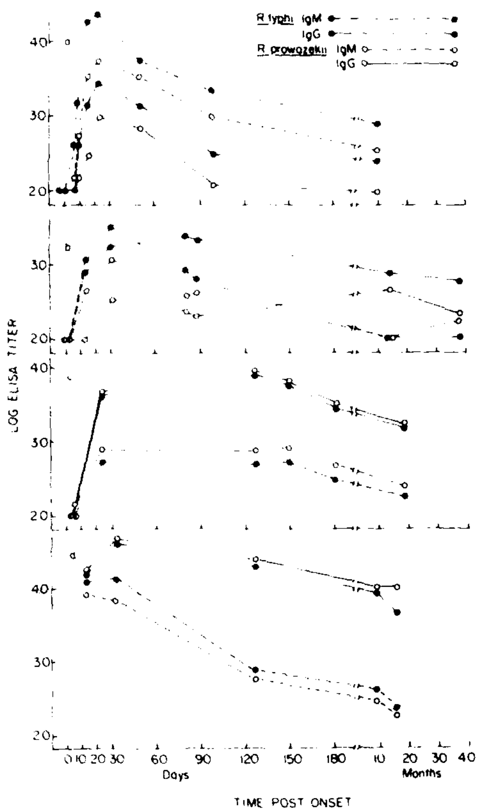

FIG. 2. *Patterns of antibody response to typhus group rickettsial infections. ELISA endpoint titers were determined as an ELISA OD of 0.25 above controls without serum, calculated by interpolation. (a) Prompt tetracycline treatment; (b) relapse on day 12 to 13 postonset of illness; (c) previous immunization with commercial (R. prowazekii) typhus vaccine; (d) delayed antibiotic treatment.*

ettsiae which we have developed (5, 10) were previously shown to be reliable methods with animal sera and selected human sera. We have now applied the procedure with equal success to a much larger sample of human sera obtained over a period of 4 years. At the screening dilution of 1:500, no known clinical illnesses were missed, and a very low rate of false positives (<1%) was obtained. Although some exposures to typhus antigen, particularly old vaccinations, were not detected (see Bourgeois et al. [2] for several cases not described here), screening at 1:100 resulted in a very high cutoff for positivity (mean ± 3 standard deviations = 0.47 OD), particularly in acute sera from various illnesses of unknown etiology. These high responses could not be attributed to rheumatoid facto. (7, 14), as assayed by the Difco latex test, and did not occur with scrub typhus antigens.

Both the ELISA results reported here (Table 2) and results by the micro-IF procedure of Ormsbee et al. (19) suggest that very few sera positive for typhus antibodies are missed if only the IgG conjugate is used in screening. The virtue of the IgM conjugate is that one can readily distinguish primary infections from the dominant IgG anamnestic responses found in Brill-Zinsser disease (17, 18) or after vaccination. The ELISA (Fig. 2c) is clearly as suitable as the micro-IF procedure (19, 20) for this purpose. Although the total lack of an IgM response is well documented in some Brill-Zinsser sera (19), Philip et al. (21) found occasional IgM responses to infection after vaccination. We found significant IgM levels in two individuals (sera 3 and 4, Fig. 1) and a low transient rise in a third (Fig. 2c), all of whom had received Cox-type epidemic typhus vaccine some years previously. However, the IgG response was predominant in each case.

The sera of the laboratory workers, who had been exposed to both *R. typhi* and *R. prowazekii*, was clearly group reactive in the typhus microplate ELISA, but small reproducible differences in species reactivities of the sera were obtained. The greater ELISA antibody responses correlated very well with the histories of those infections that were clearly defined on epidemiological grounds. These conclusions are being further evaluated with an ELISA based on the major species-specific antigens of *R. typhi* and *R. prowazekii* (G. A. Dasch, J. R. Samms, and J. C. Williams, submitted for publication).

One of the advantages of the ELISA, as performed in this study, is the clarity of definition of the humoral response to typhus antigens in different clinical situations (Fig. 2). Wisseman et al. (36) described three laboratory infections treated with regimens of chloramphenicol which did not prevent relapses and which required a second course of chemotherapy. They resembled the case illustrated in our Fig. 2b and, in one instance of delayed treatment, Fig. 2d. The presence of moderately high levels of antibodies did not prevent relapse, supporting the suggestion that cell-mediated immunity plays an important role in defense against typhus rickettsiae (16, 35). Our studies indicate that relapse or delayed treatment results in humoral responses that are primarily IgG. These reactions contrast with the predominantly IgM responses of individuals treated with tetracycline long enough to prevent early relapse (Fig. 2a). The protective value of a predominantly IgM response is not known, but the lymphocytes of two individuals with antibody responses like that depicted in Fig. 2a could be stimulated specifically by typhus antigens (2). It can be surmised, therefore, that prompt and vigorous chemotherapy may not interfere with the development of cell-mediated immunity.

The modification of the serological response to infection with murine typhus by previous immunization with epidemic typhus has been investigated previously (9, 21, 24). In both the previous studies and the present case (Fig. 2c), no consistent pattern of murine or epidemic antibody specificity was apparent after infection.

As the ELISA test was performed in this study, with a 1:500 dilution of serum, antibodies were not detected before day 7 postonset. These results compare favorably with those of Plotz et al. (23) and Scoville et al. (29), using the complement fixation test. Serological confirmation of rickettsial infection by ELISA can be made more sensitive by reducing the serum dilution to 1:100 or 1:200 and judging the reaction by rising titers rather than by any predetermined OD cutoff point. Alternatively, the versatile ELISA may prove to be a highly sensitive approach to the detection of rickettsial antigens present in body fluids or tissues early after the onset of rickettsial illness (8, 37).

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